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# Monitoring the dynamics of Src activity in response to anti-invasive dasatinib treatment at a subcellular level using dual intravital imaging

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# Monitoring the dynamics of Src activity in response to anti-invasive dasatinib treatment at a subcellular level using dual intravital imaging

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**Optimising response to tyrosine kinase inhibitors in cancer remains an extensive field of research. Intravital imaging is an emerging tool, which can be used in drug discovery to facilitate and fine-tune maximum drug response in live tumors. A greater understanding of intratumoural delivery and pharmacodynamics of a drug can be obtained by imaging drug target-specific fluorescence resonance energy transfer (FRET) biosensors in real time. Here, we outline our recent work using a Src-FRET biosensor as a readout of Src activity to gauge optimal tyrosine kinase inhibition in response to dasatinib treatment regimens *in vivo*. By simultaneously monitoring both the inhibition of Src using FRET imaging, and the modulation of the surrounding extracellular matrix using second harmonic generation (SHG) imaging, we were able to show enhanced drug penetrance and delivery to live pancreatic tumors. We discuss the implications of this dual intravital imaging approach in the context of altered tumor-stromal interactions, while summarising how this approach could be applied to assess other combination strategies or tyrosine kinase inhibitors in a preclinical setting.**

**Keywords:** adhesion, ECM, FRET, invasion and metastasis, *in vivo* imaging, pharmacodynamics, Src

**Abbreviations:** ECM, extracellular matrix; ECFP, enhanced cyan fluorescent protein; FLIM, fluorescent lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; PDAC, pancreatic ductal adenocarcinoma; SH2, Src-homology-2; SHG, second harmonic generation; RTK, receptor tyrosine kinase.



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## Introduction

Optimal drug delivery into solid tumors can be impaired by several factors[1,2] Deregulation of the extracellular matrix (ECM) composition of the tumor microenvironment is a major determinant controlling drug penetrance. The increased deposition of ECM and subsequent fibrosis in pancreatic ductal adenocarinoma (PDAC) can limit drug perfusion into the tumor mass.[3] This process in pancreatic cancer is governed by tumor-associated stellate cells, which produce excessive amounts of collagen type I and other ECM components, such as fibronectins, leading to restricted drug delivery within the tissue.[4,5] Due to its fibrillar structure collagen type I can be visualized using multi-photon intravital microscopy. Its compact molecular structure generates a second harmonic generation (SHG) signal when excited with high energy pulsed laser.[6] This signal can then be further quantified[7] and the manipulation and efficacy of anti-ECM treatment regimens can be evaluated by live intravital imaging.[8]

Recently, we demonstrated a correlation between Src expression levels and reduced survival in human pancreatic cancer, and showed that the activity of Src is an indicator of invasion and poor prognosis in human pancreatic cancer patients.[9] Importantly, we also established that the phase II, small molecule Src inhibitor, dasatinib, which is currently being clinically evaluated in combination with chemotherapy in locally advanced PDAC,[10] inhibited invasion of primary PDAC cells generated from the *Kras*$^{G12D/+}$, *Trp53*$^{R172H/+}$, *Pdx1-Cre* (KPC) model of pancreatic cancer, and reduced the development of metastases by approximately 50%.[11-13] In order to visualize Src activity upon treatment and inhibition with dasatinib within single cancer cells, we employed a FRET-based Src reporter in cells generated from this model to map the pharmacodynamics of the Src inhibitor dasatinib *in vivo* (**Fig. 1**).[8,9,12,14]

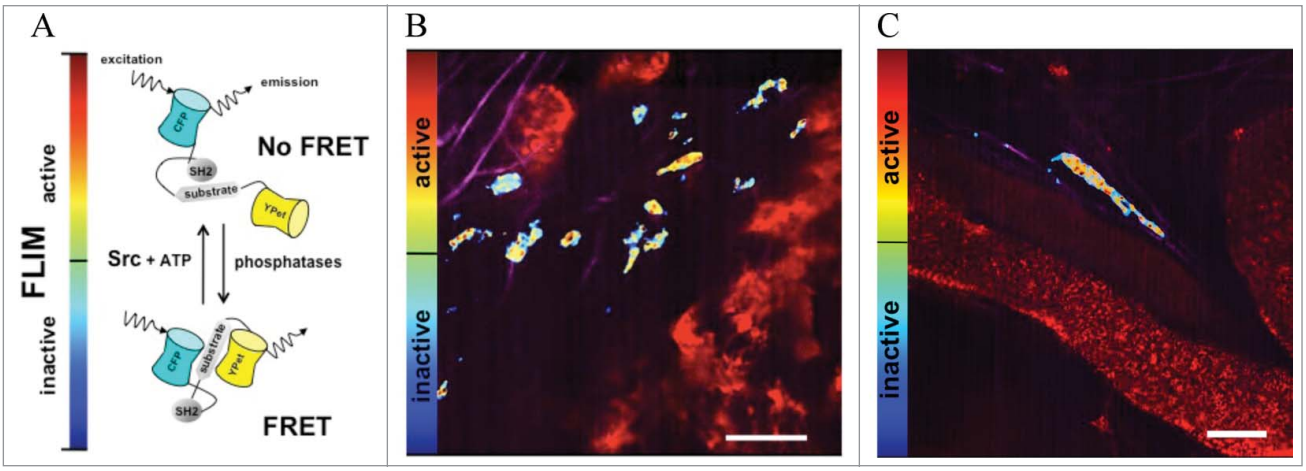


**Figure 1.** FRET-based Src biosensor *in vivo*. (**A**) Functional schematic of the Src biosensor in both a FRET (bottom) and non-FRET confirmation (top). The probe is phosphorylated by active Src and dephosphorylated by phosphatases within the cell. The activity of Src is represented by the FLIM-bar, denoting Src-active (red to yellow) and -inactive colors (green to blue). (**B and C**) Src activity distribution in single cells at subcellular resolution in relation to local vasculature *in vivo*, with vasculature in red (quantum dots, Qtracker655), collagen in magenta (detected by SHG imaging) and cells in the FLIM colors; Scale bars in (**B and C**), 50 μm.



The Src reporter consists of an SH2 domain, a flexible linker and a peptide sequence derived from the c-Src substrate p130cas, flanked by 2 spectrally overlapping fluorophores ECFP and YPet.[14] When the substrate of Src kinase is unphosphorylated, the fluorophores remain in close proximity, leading to high FRET efficiency. Upon Src-induced phosphorylation, the substrate peptide binds to the SH2 domain and separates YPet from ECFP, thereby decreasing FRET efficiency (**Fig. 1A**). This can be quantified by measuring the fluorescent lifetime (FLIM) of ECFP, which decreases when the reporter assumes a FRET-conformation. FLIM-FRET signals are then represented in heat-maps, where red to yellow colors are attributed to Src activity and blue to green colors represent inactive Src (**Fig. 1B and C**).

This application of live functional imaging of Src activity with the use of FLIM-FRET allowed us to gauge Src activity and drug treatment response with high spatiotemporal resolution in a complex setting. We assessed the temporal inactivation of Src in response to dasatinib in live tumor tissue and were able to observe and quantify a drug clearance rate post oral administration (**Fig. 2A**). Src was inactive at 2 hours post-dasatinib treatment and a gradual reactivation was observed after 4–6 hours, peaking and overcompensating at 16 hours before returning to control levels after 24 hours. We then assessed spatial regulation by examining Src activity in distinct locations within a complex microenvironment. Three-dimensional organotypic assays showed increased Src activity in proportion to PDAC invasive status (**Fig. 2B**,[8,15]). Similarly, this gradient also exists within live tumors, where Src activity is enhanced at invasive borders, relative to the tumor center, correlating with metastasis in this model.[8,9] Importantly, the organization and deposition of ECM components correlated with Src activity *in vivo*, where a highly organized, dense ECM was associated with high Src activity (quantified by SHG imaging and Gray Level Co-occurrence Matrix (GLCM) analysis, as previously described[7,8,16]). Further analysis revealed that Src regulation in response to dasatinib was also governed by the proximity of cells to the host vasculature (shown using quantum dots (**Fig. 3A**, red)). Cells distal to the vasculature (>100 μm) were less responsive to dasatinib treatment than those close to blood vessels (**Fig. 3B**, <25 μm). This detailed spatiotemporal mapping of drug performance in the context of vascularisation *in vivo*, combined with the capacity to simultaneously track ECM integrity, allowed us to utilize this technology to monitor improvements in dasatinib delivery at a subcellular resolution in response to ECM-targeted therapy.[8]

Targeting the ECM architecture to enhance drug penetration has recently been employed to improve chemotherapeutic drug delivery in solid tumor types such as pancreatic cancer. In particular, the role that stromal tissue plays in the perfusion deficit found in pancreatic cancer has been a rapidly evolving area of research.[17-19] In the KPC pancreatic mouse model described here, treatment with the hedgehog signaling inhibitor, IP-926, to deplete the tumor-associated ECM, increased tumor perfusion and the therapeutic index of standard-of-care chemotherapeutic agents such as gemcitabine resulting in increased overall survival.[19] Similarly, the enzymatic depletion of ECM glycosaminoglycans has recently been shown to improve the intratumoural delivery and efficacy of gemcitabine *in vivo*.[17,18] These studies therefore suggested that the current failure of drugs in the treatment of solid tumors may arise, partly, from a potentially reversible impairment in intratumoural drug delivery. We therefore determined whether a similar strategy could be adopted for dasatinib treatment *in vivo*.[9,10]

In order to make the inherently dense tumor mass more accessible to dasatinib treatment, tumors were pre-treated with the hedgehog signaling inhibitor,

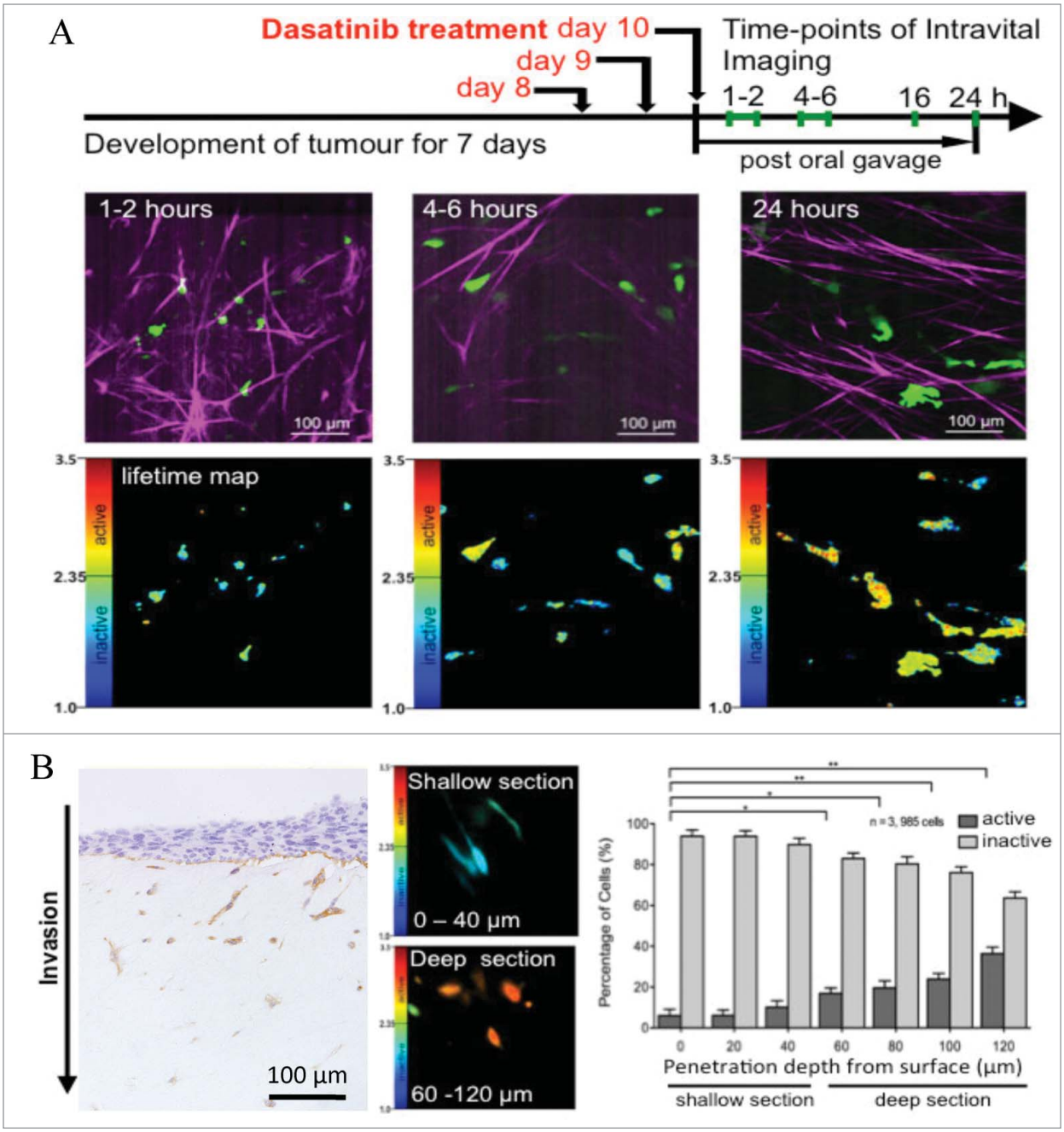


**Figure 2.** Monitoring targeted response to dasatinib *in vivo* and *in vitro* using FLIM-FRET. (**A**) Drug treatment regimen and imaging schedule (top) allowing for monitoring of dasatinib treatment efficacy *in vivo* over time, with collagen in magenta (detected by SHG imaging) and cells in green (middle) with corresponding lifetime maps of Src activity (bottom). (**B**) Src activity distribution in single cells in relation to penetration depth through collagen matrix *in vitro*, assessed by immunohistochemistry for phosphorylated Src (left, pSrc positive cells stain brown) or by FLIM-FRET with corresponding quantification at 20 µm resolution steps (right). Columns, mean; bars, standard errors; *, $p = 0.027$; **, $p = 0.021$; ***, $p = 0.006$; ****, $p < 0.001$ by unpaired Student *t* test. Figure, partially reproduced from original highlighted paper ([8] courtesy of Cancer Research).

cyclopamine, to reduce ECM content.[19,20] Manipulation of the surrounding ECM levels with cyclopamine and maintenance of tissue integrity was monitored by SHG imaging in conjunction with simultaneous FLIM-FRET imaging of the Src reporter.[8] A significant improvement in Src inhibition was achieved throughout the tumor mass using this approach, both at the tumor border versus the center, and at sites distant from the vasculature. Interestingly, at >100 µm from the vasculature, a limit was reached whereby no further inactivation of Src could be achieved. This could potentially be due to the limit of perfusion within the tissue or could be due to hypoxic and metabolic changes in tumor cells

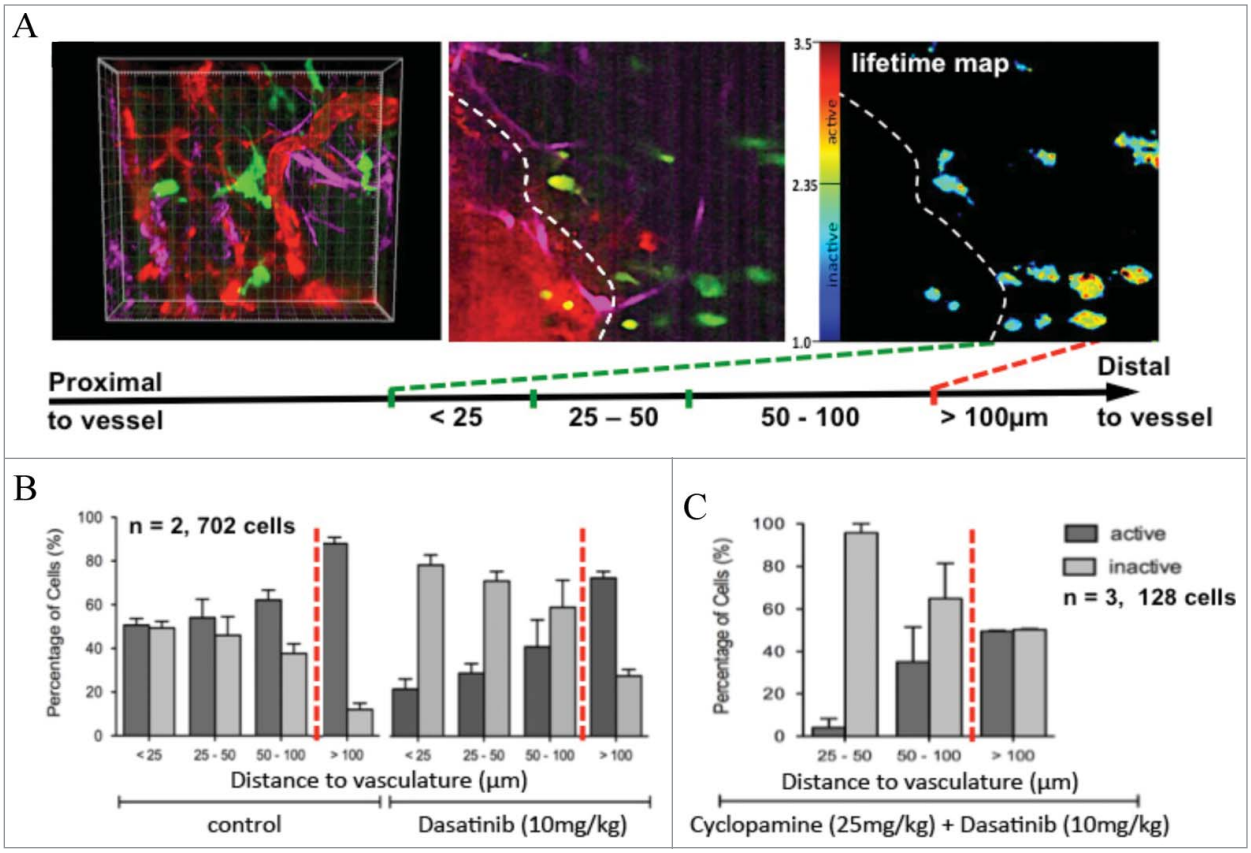


**Figure 3.** Shifting of spatial distribution of Src activity distant from local vasculature after drug treatment, as revealed by intravital imaging. (**A**) Innate gradient of Src activity distant from tumor vasculature, visualized by FLIM-FRET in conjunction with the quantum dot signal. (**B**) Quantification of shift in distribution of Src activity in single cells in relation to vessel proximity after dasatinib treatment (**C**) or after dasatinib treatment in combination with an ECM inhibitor (cyclopamine). Columns, mean; bars, standard errors. Figure, rearranged and reproduced from original highlighted paper ([8] courtesy of Cancer Research).

distant from the vasculature preventing dasatinib efficacy in this subpopulation. Further studies to assess whether this is the case are currently being carried out.

The concept that the ECM acts as a barrier to dasatinib diffusion within the tumor is unlikely to be the only explanation for the improved dasatinib response we observe in combination with ECM-targeted therapy. In **Fig.** 4, we propose that in cases of high fibrosis there is a bi-directional positive feedback loop between tumor cells and the surrounding ECM inducing Src signaling via integrin engagement, increasing the robustness of Src activity.[21,22] Upon ECM depletion or relaxation, this feedback may be reduced, thereby indirectly priming Src activity within the tumor to be more receptive to dasatinib treatment in combination therapy. In accordance with this, direct treatment of PDAC cells *in vitro*, with cyclopamine alone, had no effect on Src activity, while in the presence of signaling from the surrounding matrix *in vivo,* cyclopamine priming enhanced Src inactivation in response to dasatinib. Current studies to determine the level to which indirect ECM signaling or malperfusion plays a role in this dual targeting approach are underway.[23,24]

In this work we used a single time point to observe improved spatial inactivation of Src with combination therapy. It would be interesting for future studies to determine whether, upon ECM priming, the timing of Src inactivation occurs more rapidly or whether dasatinib accumulates and resides in the tumor for different time periods due to reduced matrix levels. Monitoring the relationships between spatial coverage and timing of drug delivery and drug retention is difficult, and this must be taken into consideration when streamlining improved drug targeting in future studies.[25]

## Targeting Other Aspects of the Tumor Microenvironment

In addition to the abundance of ECM components, tumors are heterogeneous tissues containing other elements such as blood vessels, lymphatic tissue and tumor-associated cell types such as fibroblasts, pericytes and immune cells.[26-31] The tumor microenvironment can therefore supply cancer cells with important cues during tumor progression and as is the case for the ECM, other aspects of the tumor environment could be important drug targets.

Blood vessels as transporters of oxygen, nutrients and therapeutic drugs are commonly deregulated in tumors. Their

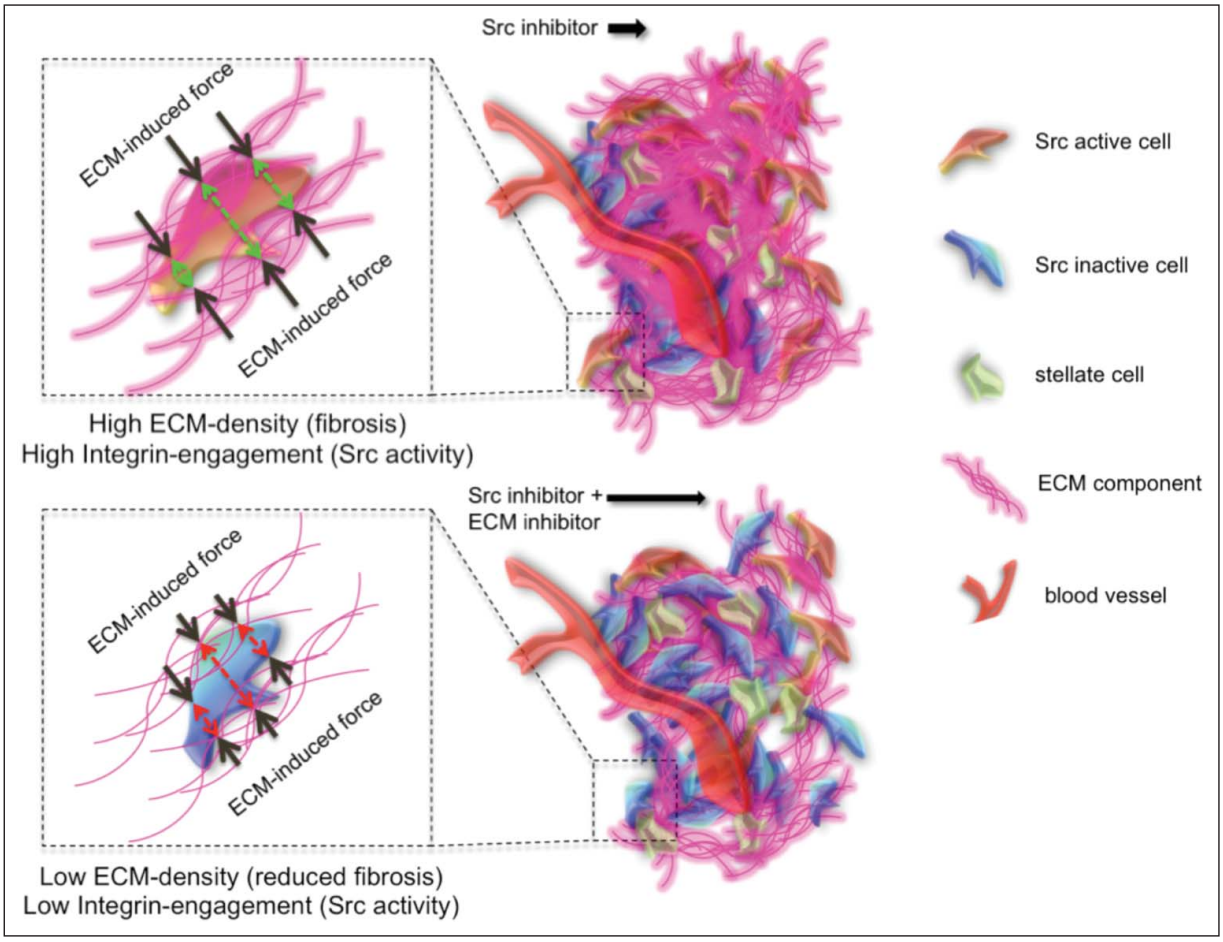


**Figure 4.** Improving drug distribution away from the vasculature by targeting the ECM and decreasing its deposition, leading to a reduction in ECM exerted force and decreased integrin engagement and thus potentially a decrease / a longer suppression of Src activity.



architecture is often poorly organized, leading to irregular blood flow, and due to the highly proliferative nature of tumor cells, blood and lymphatic vessels can become compressed within many solid tumors. As a result, this disordered composition of the vascular system often hinders drug penetrance in solid tumor types such as pancreatic cancer.[1,17,18] In line with this, recent work has demonstrated that ECM depletion reverts this compression and improves vascularity indirectly.[17,18] Another approach to enhance treatment efficacy could therefore be the indirect remodelling of the tumor vasculature. This has recently been shown with angiotensin inhibition and the associated downstream decrease in expression of pro-fibrotic signals such as transforming growth factor (TGF)-β1, connective tissue growth factor (CTGF) and endothelin (ET)-1.[32] Here, the authors show that stress exerted upon vessels by excessive deposition of collagen and ECM components in breast and pancreatic tumors was decreased by angiotensin inhibition using losartan, and thus vascular perfusion and chemotherapeutic drug delivery was increased.[32] Repurposing of angiotensin inhibitors or similar drugs in this way could therefore be used in future combination treatments to improve drug penetrance. Similarly, actively increasing vascular patency directly could also help anti-cancer agents enter the tumor tissue more readily (**Fig. 4**). To achieve higher penetration of anti-cancer agents, drugs such as fasudil[33,34] or ascorbate[35] could be used, which have been shown previously to directly increase the patency of vessels. Monitoring and maximizing these direct or indirect improvements of drug targeting combinations by FLIM-FRET analysis may be used in future preclinical settings to fine-tune such drug targeting combinations.[8,36,37]

Conversely, many tumor types are often associated with poor vasculature and vascular leakage, which impairs the efficiency of intratumoural blood pressure to drive effective drug delivery to the entire tumor. Targeting the vasculature using anti-angiogenics could help to improve drug delivery and retention in this scenario by normalization of the vessels, leading to a reduced number of leaky vessels and improving the intratumoural blood pressure of the existing vasculature (**Fig. 5**). Recently, the VEGF receptor tyrosine kinase inhibitor axitinib allowed for a longer retention time of an anti-cancer pro-drug within tumors to improve cyclophosphamide

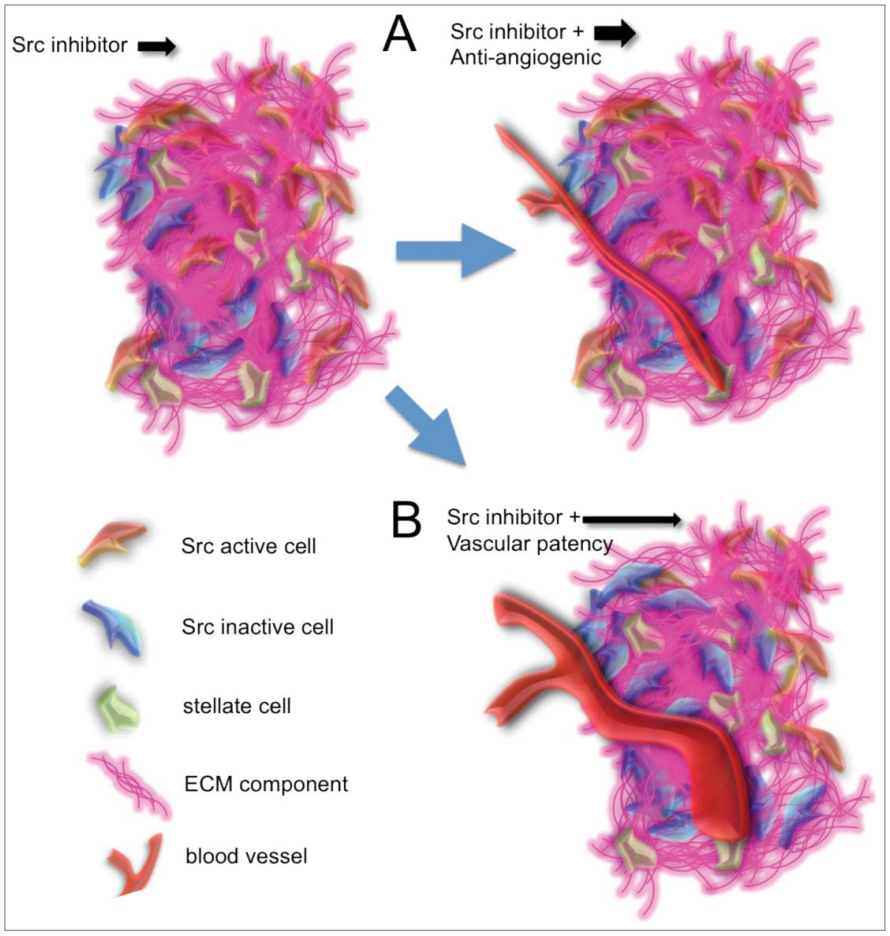


**Figure 5.** Targeting the tumor vasculature to increase drug treatment efficacies. (**A**) Application of a potential anti-angiogenic leading to vessel normalization prior to treatment with the Src inhibitor could lead to an increase of drug retention time within the tumor mass. (**B**) An increase in vascular patency could lead to an increase in the potential influx of the Src inhibitor.

cytotoxic activity in gliosarcoma xenografts.[38] This blood vessel stabilization approach has also previously been observed for the treatment of tumors with the anti-angiogenic antibodies DC101 or bevacizumab as monotherapies.[39,40] These effects, however, were only transiently obtained and thus a careful scheduling of pre-treatment of tumors with anti-angiogenics in combination with other anti-cancer agents is key for their potential future clinical success. In order to optimally induce the normalization of the vasculature at the right time of treatment and thus maximise the retention of the drug in question, we propose that live intravital imaging of target inactivation could be employed in conjunction with monitoring blood vessel changes and permeability using quantum dots or fluorescent dextrans, as recently achieved.[8,37,41-43]

Our application of matching the drug of choice with available target reporters opens up new possibilities for precise monitoring of spatiotemporal signaling in drug discovery. Here, we matched dasatinib treatment with a Src-FRET reporter in a model of invasive pancreatic cancer in which Src inhibition has previously shown promise in both animal models and current phase II clinical trials.[9,10] However, FRET reporters of different targets in other models of disease or drug treatments could also be applied in a comparable fashion (**Fig. 6**, drug A matched with target A). Similarly, indirect readout of downstream targets could also be achieved using this setup (**Fig. 6**, drug B). For example, Src activity downstream of EGFR targeting via gefitinib could be assessed in the current setup and may provide additional detail to the signal transduction cascade in receptor tyrosine kinase (RTK)-driven disease states in response to drug treatment.[44,45] Expression of multiple FRET biosensors within the same cell, which do not spectrally overlap, are also becoming available and could provide distinct parallel or antagonistic information in response to single agent treatments (**Fig. 6**, drug C,[46-49]). This spatiotemporal approach could also be used to monitor signaling feedback loops to dissect redundant or resistance mechanisms in response to drug treatment (**Fig. 6**, drug C,[50]). Moreover, targeting of stromal cell types, such as fibroblasts or immune cell populations within tumors, could also be assessed for paracrine interactions with cancer cells and indirect targets could be monitored with fine temporal and spatial resolution using this approach (**Fig. 6**, drug D).

Furthermore, this application in subcutaneous xenograft tumors lacks the inherent host environment unique to the tissue type in question. Our latest genetic engineering of a FRET reporter mouse,[51] crossed to pancreatic, mammary and intestinal tumor models has recently allowed us to apply this approach to native tissue and could provide significant insight into how molecular targets commonly hijacked in cancer behave in more physiological and functional settings in the future.[51] Finally, the use of cutaneous or abdominal optical windows[52–54] permits longitudinal monitoring of protein activity *in vivo* in the same animal over repeated imaging periods and may further advance the screening of drug targeting efficacy in this setting.

In conclusion, the capacity to monitor changes in drug targeting at the molecular level in the context of the tumor landscape, including the proximity of cells to the host vasculature, their location with regards to tumor invasive borders and the integrity of the surrounding ECM or blood supply, could provide unprecedented insight into how best to streamline drug targeting within live tumor environments.

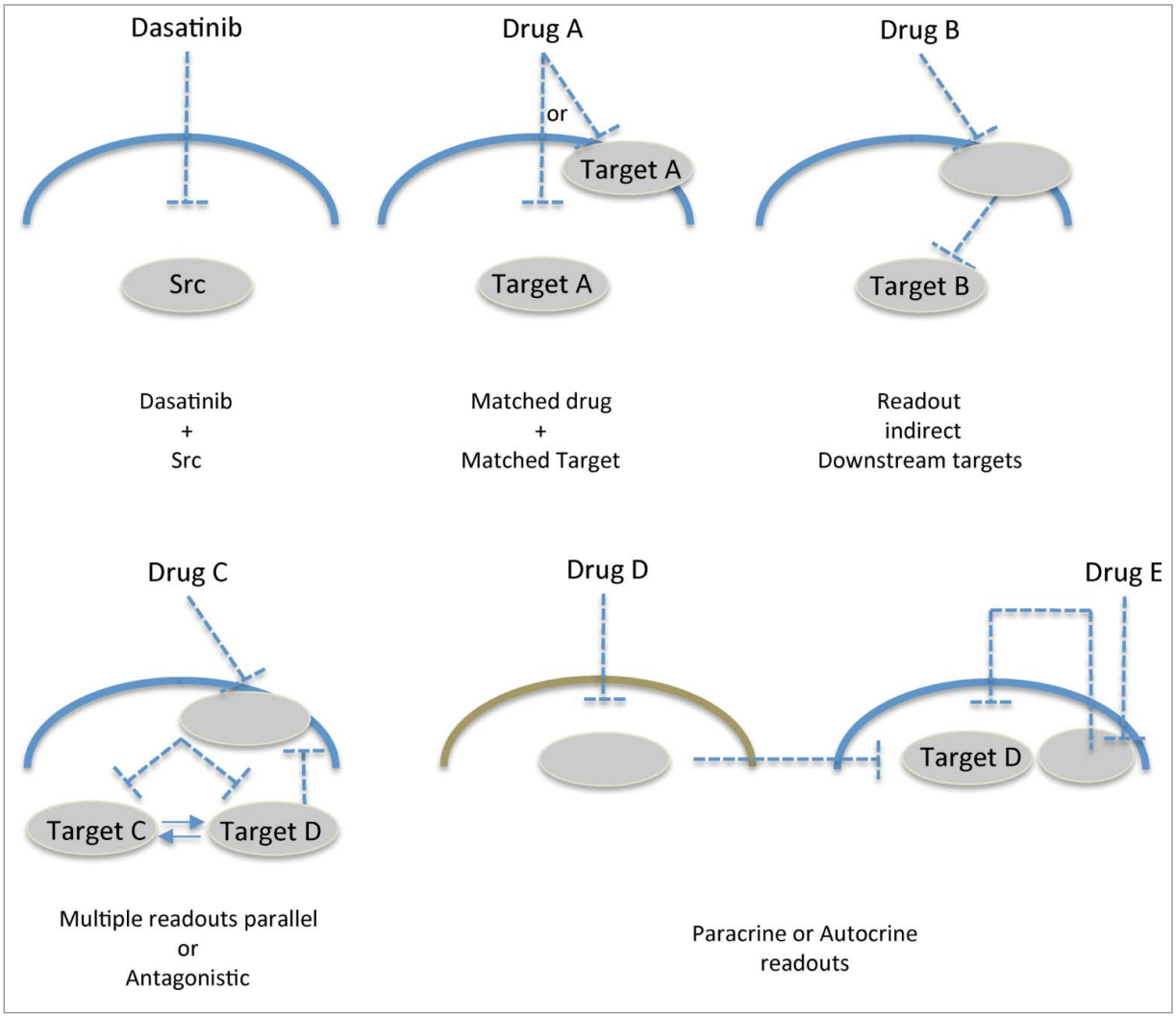


**Figure 6.** Schematic of potential applications of intravital FRET imaging. Depicting the current highlighted study, dasatinib matched with Src reporter, drug A with reporter and target **A**, drug (**B**) with indirect reporter and target **B**, drug (**C**) with downstream reporters and targets (**C or D**). Drug (**D**) in other tumor-associated cell types (e.g. fibroblasts, immune cells) providing indirect paracrine read out of the tumor cells of target D or read out of drug E by autocrine signaling inhibition of target (**D**).



### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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